A lectin from *Bothrops leucurus* snake venom raises cytosolic calcium levels and promotes B16-F10 melanoma necrotic cell death via mitochondrial permeability transition


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**ABSTRACT**

BIL, a galactose-binding C-type lectin purified from *Bothrops leucurus* snake venom, exhibits anticancer activity. The current study was designed to elucidate the cellular mechanisms by which BIL induces melanoma cell death. The viabilities of B16-F10 melanoma cells and HaCaT keratinocytes treated with BIL were evaluated. Necrotic and apoptotic cell death, cytosolic Ca\(^{2+}\) levels, mitochondrial Ca\(^{2+}\) transport and superoxide levels were assessed in B16-F10 melanoma cells exposed to BIL. We found that treatment with BIL caused dose-dependent necrotic cell death in B16-F10 melanoma cells. Conversely, the viability of non-tumorigenic HaCaT cells was not affected by similar doses of BIL. BIL-induced B16-F10 necrosis was preceded by a significant (2-fold) increase in cytosolic calcium concentrations and a significant (3-fold) increase in mitochondrial superoxide generation. It is likely that BIL treatment triggers B16-F10 cell death via mitochondrial permeability transition (MPT) pore opening because the pharmacological MPT inhibitors bongkrekic acid and Debio 025 greatly attenuated BIL-induced cell death. Experiments evaluating mitochondrial Ca\(^{2+}\) transport in permeabilized B16-F10 cells strongly supported the hypothesis that BIL rapidly stimulates cyclosporine A-sensitive Ca\(^{2+}\)-induced MPT pore opening. We therefore conclude that BIL causes selective B16-F10 melanoma cell death via dysregulation of cellular Ca\(^{2+}\) homeostasis and Ca\(^{2+}\)-induced opening of MPT pore.

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**1. Introduction**

Snakes from the *Bothrops* genus are responsible for more than 20,000 accidents per year in Brazil, totaling nearly 90% of all recorded snakebites. *Bothrops leucurus* mainly inhabits the northeast region of Brazil, and its venom elicits biological effects that are typical of the *Bothrops* genus, which include coagulative, fibrinolytic and hemorrhagic effects, acute renal failure and local necrosis (de Morais et al., 2013).

*B. leucurus* venom is composed of a complex mixture of bioactive substances, mainly peptides and proteins that are capable of interfering with a variety of physiological processes. A protein named BIL, which is a galactose-binding C-type lectin that is purified from *B. leucurus* snake venom, exhibits anticancer and immune-related activities (Nunes...
Edos et al., 2011, Nunes et al., 2012) that are of biomedical interest. BL is a dimeric protein composed of two 15 kDa subunits and has been shown previously to elicit antibacterial activity against human pathogenic gram-positive bacteria (Nunes Edos et al., 2011) and to cause cytotoxic effects in human tumor cell lines (Nunes et al., 2012). The latter action of BL was associated with phosphatidyserine externalization and disruption of the inner mitochondrial membrane potential (Nunes et al., 2012). Other studies have indicated that lectins such as concanavalin A, Polygonatum odoratum, Polygonatum cyrtomena and mulberry leaf lectin also promote cell death through mechanisms involving interactions with mitochondrial membranes (Liu et al., 2009a, 2009b, 2009c; Zhao et al., 2010).

In general, lectins are proteins that are able to recognize and bind to specific carbohydrate domains on cell membrane. Upon lectin binding to plasma membrane receptor and/or internalization by endocytosis, a variety of cellular events and lectin interaction with intracellular organelles may arise (Lichtenstein and Rabinoivich, 2013). We have recently found that a lectin purified from Craytilia mollis seed, Cramoll 1,4, which belongs to the mannose/glucose binding class of lectins, promotes Trypanosoma cruzi epimastigote parasite necrosis via a concerted action on plasma and mitochondrial membranes leading to mitochondrial Ca$^{2+}$ overload and stimulation of reactive oxygen species production followed by necrosis-like cell death (Fernandes et al., 2010). In addition, it was also found that Cramoll 1,4 promotes mitochondrial permeability transition (MPT) pore opening in isolated rat liver mitochondria (Fernandes et al., 2014). MPT is a non-selective permeabilization of the inner mitochondrial membrane (Hunter et al., 1976; Zoratti and Szabò, 1995; Crompton, 1999) that is triggered by mitochondrial calcium overload and can be followed by cell death.

Given the involvement of MPT in both apoptosis and necrosis (Crompton, 1999; Figueira et al., 2013), the development of pharmacological agents able to modulate MPT pore opening is of great interest, as are the potential development of pharmacological agents able to modulate necrosis (Crompton, 1999; Figueira et al., 2013), the and can be followed by cell death.

In this work we obtained from Sigma (St. Louis, USA). Annexin V-FITC and propidium iodide (PI) were obtained from Invitrogen (Carlsbad, USA). All other reagents used in this work were obtained from Sigma (St. Louis, USA).

2.2. Lectin preparation

B. leucus venom was kindly supplied by the Nucleo Regional de Oﬁologia e Animais Peçonhentos da Bahia, Universidade Federal da Bahia, Salvador, Bahia, Brazil. BL was purified according to the protocol previously described by Nunes Edos et al. (2011). Briefly, lyophilized crude venom of B. leucus (30 mg) was dissolved in 1 mL of CTBS buffer (20 mM Tris-HCl, 150 mM NaCl and 5 mM CaCl$_2$, pH 7.5) and centrifuged (2000 g, 5 min, 25 °C) to remove insoluble material. The resulting supernatant was applied to a column (10 × 1.0 cm) of guar gel previously equilibrated with CTBS at a flow rate of 10 mL/h. BL was eluted from the column with 200 mM galactose in CTBS.

2.3. Cell culture and treatments

B16-F10 melanoma cells were obtained from American Type Culture Collection (ATCC, Virginia, USA) and grown in RPMI-1640 (Vitrocell, São Paulo, Brazil) supplemented with 10% fetal bovine serum, 100 μg/mL gentamicin, 100 IU/mL penicillin and 100 μg/mL streptomycin (Vitrocell, São Paulo, Brazil). HaCaT cells, a keratinocyte cell line that was purchased from Cell Line Service (CLS, Heidelberg, Germany), were maintained in DMEM with high glucose (Vitrocell, São Paulo, Brazil) supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 U/mL penicillin and 100 μg/mL streptomycin. The cells were maintained at 37 °C in a humidified atmosphere with 5% CO$_2$.

Analyses of cell viability and death were conducted after 24 h exposure to BL at varying concentrations. Then, to evaluate cellular events and the mechanisms leading to cell death, mitochondrial superoxide levels, cytosolic Ca$^{2+}$ levels and cytoprotection by MPT inhibitors were assessed in a time frame of 6–12 h after the exposure of cells to BL. Treatment periods are specified in figure legends.

2.4. BL effects on B16-F10 and HaCaT cell viability

Cell viability and proliferation were evaluated in the presence of varying concentrations of BL. The cells were trypsinized using a trypsin-EDTA solution from Vitrocell and were centrifuged at 1500 g for 4 min. The resulting cell pellet was resuspended in 3 mL of RPMI-1640 media for B16-F10 or DMEM for HaCaT; both media included 10% fetal bovine serum. Cell viability was assessed using trypan blue dye (0.1%) added to aliquots of cell suspensions, and the percentages of stained cells were determined microscopically. Cell viability under control condition was above 95%.

2.5. Flow cytometry analysis of cell death

The percentage of apoptotic and necrotic cells was determined using a FACSCalibur flow cytometer (BD Biosciences, USA) equipped with an argon laser and Cell-Quest software (version 4.1). For the analysis, 10$^6$ cells were incubated in labeling buffer (10 mM HEPES, pH 7.4, 150 mM NaCl, 5 mM KCl, 1 mM MgCl$_2$ and 1.8 mM CaCl$_2$) containing annexin V-FITC (1:500) and PI (20 μg/mL) at room temperature for 20 min in the dark. Ten thousand events were acquired and apoptosis was quantified by as the number of annexin V-FITC-positive and PI-negative cells divided by the total number of cells, while necrosis was quantified as the number of PI-positive and annexin V-FITC-negative cells divided by the total cell number.
2.6. Microscopy

B16-F10 melanoma cells were treated with BIL at various concentrations for 24 h. Then, the B16-F10 cells were photographed under a Leica DFC360 FX microscope using LAS AF software (Leica Microsystems, Wetzlar, Germany).

2.7. Measurement of mitochondrial superoxide levels

Following the treatment of B16-F10 cells with BIL for 6 h, the cells (10⁶) were incubated in RPMI medium with 5 μM MitoSOX at 37 °C for 10 min to detect mitochondrial superoxide levels. MitoSOX fluorescence intensity, which indicates mitochondrial superoxide levels, was analyzed by flow cytometry (FACSCalibur, BD Biosciences, San Jose, USA).

2.8. Spectrofluorimetric determination of cytosolic Ca²⁺ levels

Following the treatment of B16-F10 cells with BIL for 6 h, the cells (10⁶) were washed twice at 6000 g for 4 min at 4 °C in PBS containing 11 mM glucose and 0.8 mM MgSO₄, pH 7.2. Cells were resuspended in PBS containing 11 mM glucose and 0.8 mM MgSO₄, pH 7.2 and 5 μM Fura 2-AM (Sigma, New York, USA). The suspensions were incubated for 45 min and maintained at 37 °C in a humidified atmosphere with 5% CO₂. Subsequently, the cells were washed with PBS to remove extracellular dye. Cells were resuspended to a final density of 10⁶ cells/mL in PBS. The ratiometric fluorescence of this suspension was recorded in a cuvette placed into a thermostatically regulated (37 °C) F-4500 Hitachi spectrofluorometer (Japan). The spectrofluorometer was set to excite the probe at 340 and 380 nm and to read the emission at 510 nm. The Fura 2 fluorescence response was calibrated from the ratio of 340/380 nm fluorescence values after subtraction of the background fluorescence of the cells at 340 and 380 nm as described by Gryniewicz et al. (1985) and Vercesi et al. (1993).

2.9. Mitochondrial Ca²⁺ transport and mitochondrial permeability transition opening

Changes in free Ca²⁺ concentrations in suspensions of digitonin-permeabilized cells were followed by measuring the fluorescence of Calcium Green-5N (Invitrogen, Carlsbad, CA) recorded on an F-4500 Hitachi spectrofluorometer operating at 506 and 532 nm for excitation and emission, respectively.

2.10. Statistical analysis

Differences among groups were assessed by Student’s t-test. The significance level was set at P < 0.05. All tests were performed using the software SigmaStat 3.1 (Systat, San Jose, CA, USA). Data are presented as means ± standard errors of the mean (SEM).

3. Results

3.1. BIL induces necrosis in B16-F10 cells but not in HaCaT cells

BIL’s effects on B16-F10 cell viability were evaluated using the trypan blue exclusion test and flow cytometry after 24 h of exposure to varying concentrations of the lectin. Fig. 1A shows the progressive decrease in the

![Fig. 1](image-url)
number of viable B16-F10 cells as BIL concentrations were increased from 50 to 100 μg/mL. A decrease in the viable cell number of nearly 50% (47.2 ± 5.6, P < 0.01) was observed at 75 μg/mL (Fig. 1A), along with a decrease in cell viability (Fig. 1B). Flow cytometry analyses of cell death similarly indicated an IC50 (49.1 ± 6.7%) of 75 μg/mL (Fig. 2A). The ratio of PI-positive cells to the total number of cells (i.e., the necrotic index) was 38.6 ± 2.7% (Fig. 2A). We verified alterations in the cells upon treatment with BIL using optical microscopy (Fig. 1C). These images evidence the decrease in total cell count and the cellular agglutination promoted by this lectin. In contrast, BIL treatment of HaCaT keratinocytes, a non-tumorigenic cell line, did not cause significant changes in cell viability as analyzed by flow cytometry (Fig. 2B).

3.2. BIL treatment increases both [Ca2+]cyt and mitochondrial superoxide anion levels in B16-F10 melanoma cells

To better understanding the mechanisms involved in the necrotic cell death elicited by BIL treatment, we evaluated the cytosolic levels of free calcium ([Ca2+]cyt) and mitochondrial superoxide anions after 6 h of B16-F10 cells incubation with BIL. This assay revealed that BIL treatment led to a 108.6 ± 32.8% increase in [Ca2+]cyt (P < 0.02) (Fig. 3) and a three-fold increase in the mitochondrial superoxide anion levels (Fig. 4).

3.3. Effect of BIL on Ca2+ movements in permeabilized B16-F10 melanoma cells

Fig. 5 shows that addition of Ca2+ to a reaction medium containing the fluorescent probe Calcium Green-5N promotes a prompt increase in the fluorescence signal. The subsequent addition of digitonin to permeabilize the plasma membrane was followed by mitochondrial Ca2+ uptake, as evidenced through the release of the accumulated Ca2+ induced by the respiratory inhibitor antimycin A (Fig. 5, trace a). The subsequent addition of the Ca2+ ionophore ionomycin caused the release of only a small fraction of the cellular Ca2+, thus supporting the...

![Fig. 2.](image-url) BIL induces necrosis in B16-F10 cells (A) but not in HaCaT cells (B). Both cell lines were treated with varying concentrations of BIL for 24 h. The percentages of necrotic (PI+), apoptotic (AnxV−) and alive (PI−AnxV+) cells were determined using flow cytometry. Values are the means ± S.E.M. of at least five independent experiments. *Significantly different from control at P < 0.05, Student’s t-test.

![Fig. 3.](image-url) Treatment with BIL (75 μg/mL) increases [Ca2+]cyt in B16-F10 melanoma cells. B16-F10 cells were treated with BIL for 6 h and then probed with 5 μM Fura 2-AM to measure cytosolic Ca2+ levels. Values are the means ± S.E.M. of at least four independent experiments. *Significantly different from control at P < 0.02, Student’s t-test.

![Fig. 4.](image-url) Treatment of B16-F10 melanoma cells with BIL (75 μg/mL) increases mitochondrial superoxide levels. Following 6 h of treatment with BIL, cells (10⁶/mL) were incubated with 5 μM MitoSOX at 37 °C for 10 min. Superoxide levels were then analyzed by flow cytometry. Values are the means ± S.E.M. of at least five independent experiments. *Significantly different from control at P < 0.05, Student’s t-test.

![Fig. 5.](image-url) BIL acutely impairs mitochondrial Ca2+ uptake and retention in a manner that is sensitive to cyclosporine A. B16-F10 melanoma cells (10⁶ cells/mL) were added to standard reaction medium containing 0.2 μM Calcine Green-5N to probe for extra-mitochondrial Ca2+ levels and then permeabilized with digitonin to allow for in situ study of mitochondrial Ca2+ transport. Where indicated, the following reagents were added to all experimental conditions: 20 μM Ca2+, 15 μM digitonin (Dig.), 5 μM antimycin A (AA) and 10 μM ionomycin (Iono). The experimental conditions were as follows: trace a, control; trace b, addition of 75 μg/mL BIL to the reaction medium; trace c, addition of 75 μg/mL BIL and 1 μM cyclosporine to the reaction medium. The results shown are representative of four independent experiments. a.u., arbitrary units.
interpretation that most of the initial decrease in Ca\(^{2+}\) levels was caused by accumulation of this cation in mitochondria. Interestingly, pre-incubation of B16-F10 cells in the same reaction medium containing BIL and Ca\(^{2+}\) was followed by only a transient decrease in Ca\(^{2+}\) levels after plasma membrane permeabilization by addition of digitonin (Fig. 5, trace b). This suggests that BIL decreased the ability of the mitochondria to accumulate and retain Ca\(^{2+}\). Indeed, under these conditions the addition of antimycin A was not followed by significant Ca\(^{2+}\) release. Further evidence that BIL’s effect on mitochondria is mediated by MPT was provided by the restoration of the mitochondrial capacity for Ca\(^{2+}\) uptake and retention observed when the experiment was carried out in the presence of the MPT inhibitor cyclosporine A (Fig. 5, trace c).

3.4. MPT inhibitors prevent B16-F10 melanoma cell death induced by BIL

To assess whether MPT was involved in the process of BIL-induced cell death, the cells were incubated with BIL for 12 h in the presence of the MPT inhibitor Debio 025 or the MPT inhibitor bongkrekic acid. The results of a trypan blue exclusion assay (Fig. 6A) demonstrate that both inhibitors protect against the decrease in the number of viable cells induced by BIL treatment. The flow cytometry analyses show that BIL-induced cell death was decreased by 69.8 ± 0.6% (\(P < 0.01\)) and 77.4 ± 1.8% (\(P < 0.01\)) in the presence of bongkrekic acid or Debio 025, respectively (Fig. 6B). Fig. 6C depicts representative histograms of these flow cytometry analyses showing the effects of bongkrekic acid and Debio 025 on necrosis caused by BIL treatment.

4. Discussion

The current study demonstrates that BIL causes cell death by necrosis in B16-F10 melanoma cells. Interestingly, BIL treatment did not affect the viability of HaCaT, a nontumorigenic cell line. Previous studies have indicated that lectins may allow normal cells to be distinguished from malignant cells (Sabova et al., 2010) because they bind to specific carbohydrate recognition domains present in cell membranes that are differentially expressed in tumor cells (Sharon, 2007; Koh et al., 2011; Nolte et al., 2012). As the processes of lectins internalization are important steps regulating the intracellular availability and the effects of these proteins, putative differences in the uptake of BIL by the two cell lines could also explain their differential susceptibility (Lichtenstein and Rabinovich, 2013). In this light, we have observed that a

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**Fig. 6.** B16-F10 melanoma cells death induced by BIL can be blunted by treatment with the MPT inhibitors Debio 025 and bongkrekic acid. Cells were treated with BIL for 12 h in the presence of the MPT inhibitor Debio 025 or bongkrekic acid. Cell death was assessed by flow cytometry analysis of propidium iodide and annexin V-FITC staining. **A.** Absolute numbers of viable cells are depicted. Cell viability under control condition was 96%. **B.** Relative extent of cell death. **C.** Representative traces of flow cytometry analysis of cell death under the four different experimental conditions. In A and B, the values are expressed as the means ± SEMs of six independent experiments for each condition. *\(P < 0.01\) versus control cells. **P < 0.01 versus BIL-treated cells.
plant seed lectin (Cramoll 1,4) binds to glycoproteins on the cell membrane of *T. cruzi* epimastigote parasites and is subsequently internalized and colocalizes with mitochondria (Fernandes et al., in press). Our data showed that the cytotoxicity of the plant seed lectin resulted from a concerted action on the parasite’s plasma and mitochondrial membranes leading to increased $[Ca^{2+}]_{\text{cyt}}$, followed by mitochondrial Ca$^{2+}$ overload and reactive oxygen species production (Irigoin et al., 2009; Fernandes et al., 2010). Notably, dysregulation of cellular and mitochondrial Ca$^{2+}$ homeostasis plays an important role in mitochondria-mediated cell death (Figueira et al., 2013). In this study, we detected an increase in $[Ca^{2+}]_{\text{cyt}}$ at an early time point (6 h) after incubation with BlL, while cell viability and death were observed 18 h later, suggesting that the Ca$^{2+}$ signal may have propagated to the mitochondria, where Ca$^{2+}$ overload triggers MPT pore opening. As a result of organelle failure and release of signaling factors into the cytosol, MPT pore opening can be followed by cell death. Indeed, the use of pharmacological MPT inhibitors (Debio 025 and bongkrekic acid) revealed that MPT pore opening plays a major role in BlL toxicity in B16-F10 melanoma cells (Fig. 6). Debio 025 is a non-immunosuppressant cyclosporine A analogue that targets mitochondrial cyclophilin D and inhibits Ca$^{2+}$-induced MPT pore opening (Quarato et al., 2012). Unlike cyclosporine A, Debio 025 does not inhibit the calcineurin pathway (Quarato et al., 2012). It is worth mentioning that we could not test the effect of cyclosporine A on BlL-induced cell death because this drug itself decreased cell proliferation compared to experimental controls (data not shown). A proposed sequence of cellular events leading to cell death upon BlL exposure is depicted in Fig. 7.

The effects of BlL on mitochondrial Ca$^{2+}$ homeostasis were further studied *in situ* in permeabilized B16-F10 cells (Fig. 5). This valuable approach revealed that BlL treatment promptly triggers Ca$^{2+}$-induced MPT. Ca$^{2+}$-induced MPT is a complex process whose molecular regulation is poorly understood. However, the likelihood of MPT pore opening is known to be modulated by mitochondrial nicotinamide (NAD, NADP) and adenine (ADP, ATP) nucleotides (Vercesi, 1987; Zoratti and Szabó, 1995; Crompton, 1999; Saito and Castilho, 2010; Ronchi et al., 2013) and mitochondrial proteins, such as the adenine nucleotide translocator and cyclophilin D (Baines et al., 2005). While ADP and reduced NADP are notable endogenous inhibitors of MPT pore opening, redox imbalance is the main factor that facilitates MPT pore opening *in vitro* and under a variety of pathological conditions (Crompton, 1999; Kowaltowski et al., 2001; Figueira et al., 2013). The role of cyclophilin D in MPT has received great attention; ablation or pharmacological targeting of this protein greatly desensitizes mitochondria to Ca$^{2+}$-induced MPT (Baines et al., 2005). Conversely, increased expression of cyclophilin D is associated with higher susceptibility to Ca$^{2+}$-induced MPT (Naga et al., 2007; Figueira et al., 2011). Post-translational modifications of cyclophilin D, namely cysteine nitrosylation and p53 binding, have emerged as key signaling events mediating in oxidative stress-stimulated Ca$^{2+}$-induced MPT pore opening (Nguyen et al., 2011; Vaseva et al., 2012). In the intact cells studied here, BlL was found to elevate $[Ca^{2+}]_{\text{cyt}}$ levels and cause mitochondrial oxidative imbalance in B16-F10 melanoma cells; both of these changes favor MPT pore opening. Direct effects of BlL on MPT (Fig. 5) may also contribute to the mitochondria-mediated cell death observed upon BlL treatment.

Although other studies have reported cell death upon interaction of C-type lectins with mitochondria, this is the first study directly demonstrating that MPT takes part in the toxicity of a major class of proteins broadly distributed in nature on cancer cells. We conclude that BlL-induced selective B16-F10 melanoma cell death occurs via dysregulation of cellular Ca$^{2+}$ homeostasis and opening of MPT pore.

**Ethical statement**

The experiments comply with international and local ethical standards.
Acknowledgments

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Conflict of interest

The authors declare no conflict of interest.

References


